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Human mast cells produce type VIII collagen *in vivo*

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Summary. Mast cells are assuming importance not only in their familiar role in acute allergic and parasitic diseases but also in chronic inflammatory, immunologic and fibrotic states. The processes by which human extracellular matrices are influenced by mast cells have remained obscure. We report here the production of type VIII collagen by human mast cells. Mast cells representing each of the known phenotypes were identified in a variety of tissues using histochemical techniques, and monoclonal antibodies specific for tryptase, chymase, and c-*kit*. Mast cells in normal and pathologic tissues expressed type VIII collagen α-1 chain protein and mRNA, detected by immunohistochemistry using monoclonal and polyclonal antibodies, and non-isotopic oligonucleotide *in situ* hybridization using digoxigenin-labelled oligonucleotide probes based on the published human α-1 collagen VIII sequence. Perivascular location of type VIII collagen positive mast cells was a striking finding. The secretion of type VIII collagen by mast cells *in vivo* may contribute to angiogenesis, tissue remodelling, and fibrosis.

Keywords: mast cells, type VIII collagen, fibrosis, angiogenesis, immunohistochemistry, *in situ* hybridization

There is increasing evidence that mast cells participate in angiogenesis, chronic inflammation and fibrosis (Galli 1990; 1993). Mast cell accumulation correlates with angiogenesis both for normal blood vessel growth and pathological vessel formation (Meininger & Zetter, 1992). Mast cell numbers and activity are increased in chronic murine graft versus host disease, a model for scleroderma, and in active scleroderma itself (Claman 1990; Frieri 1992; Hawkins *et al.* 1985). In rheumatoid arthritis, some forms of pulmonary fibrosis, and inflammatory bowel disease, the mast cell has been implicated in fibrosis (Bienenstock *et al.* 1986; Galli 1993). Mast-cell secreted cytokines have been proposed as important modulators of fibroblast activity (Galli *et al.* 1991). There is no evidence that human mast cells synthesize extracellular matrix components, although there are limited data that murine mast cells produce basement membrane constituents (Thompson *et al.* 1991).

Type VIII collagen is a non-fibrillar short-chain collagen originally identified in cell culture medium of bovine aortic and endothelial cells (Sage *et al.* 1980), corneal endothelial cells (Benya 1980), and several tumour cell lines (Alitalo *et al.* 1983; Sage *et al.* 1984).

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It is the principal component of the hexagonal lattice in Descemet's membrane (Yamaguchi *et al.* 1991), and is also expressed in sclera and choroid, optic nerve sheath, periosteum, perichondrium, meninges, and the subendothelial layer of blood vessels (Kapoor *et al.* 1988; Kittelberger *et al.* 1989; 1990). In angiogenesis, type VIII collagen is believed to facilitate the assembly of endothelial cords and tubes (Iruela-Arispe *et al.* 1991; Rooney *et al.* 1993), and its synthesis precedes or coincides with that of type I pro-collagen (Sage & Iruela 1990). Expression of type VIII collagen is increased in the abnormal vessels associated with human brain tumours (Paulus *et al.* 1991). In the extracellular matrix, type VIII collagen may act as a scaffold, forming a molecular bridge connecting other matrix components. In the rat kidney, for example, type VIII collagen molecules secreted by mesangial cells are thought to determine the structural organization of the subendothelium and the mesangial matrix, by polymerizing and interacting with matrix components (Rosenblum *et al.* 1993).

We examined the capacity of human mast cells to produce type VIII collagen, as both are associated with angiogenesis and fibrosis. A range of mast cell-bearing human tissues were studied by immunohistochemistry and *in situ* hybridization, using reagents specific for α-1 type VIII collagen protein and mRNA.

Materials and methods

Antibodies

Rabbit polyclonal antibody, reactive with α-1 chain of type VIII collagen, was prepared as previously described using ovine Descemet's membrane type VIII collagen as immunogen (Kittelberger *et al.* 1989). Monoclonal antibodies against purified bovine type VIII collagen, designated 6A2 and 9H3, were produced and characterized as previously described (Sawada *et al.* 1990): both are specific for the α-1 chain of type VIII collagen (Sawada & Konomi 1991). Affinity-purified goat antibodies to collagen types I, III, and V (Southern Biotechnology, Birmingham, AL, USA) and monoclonal antibodies to collagen IV (Dako Corp., Carpinteria, CA, USA), laminin-A and laminin-B2 (Life Technologies, MA, USA), s-laminin (Developmental Studies Hybridoma Bank, IA, USA) and heparan sulphate proteoglycan (Chemicon, Temecula, CA, USA) were also used in immunohistochemical experiments.

Mast cells were identified and phenotyped with monoclonal antibodies to human tryptase and chymase (Chemicon, Temecula, CA, USA) and to c-*kit* (generous gift of Dr Linda Ashman, Adelaide, Australia). Chymase and tryptase are unique to mast cells (Galli 1990), and c-*kit* is expressed only on mast cells and some haematologic progenitor and malignant cells (Lerner *et al.* 1991). All human mast cells express tryptase, but only a subset produce detectable levels of chymase: chymase-negative mast cells are referred to as the MC^T phenotype, while chymase-positive cells are designated MC^{TC} (Galli 1990; Irani *et al.* 1986). Mast cells in human tissue also express c-*kit*, the receptor for stem cell factor, although immunohistochemical staining for c-*kit* may vary from cell to cell (Mayrhofer *et al.* 1987). For double-labelling experiments polyclonal rabbit antibody against von Willebrand factor (vWf) (Dako) was used to identify blood vessel endothelium.

Tissues studied

We studied a variety of tissues bearing mast cells representing each of the known human mast cell phenotypes: urticaria pigmentosa in the skin, reactive tissue surrounding carcinoma of the kidney, asthmatic lung, idiopathic pulmonary fibrosis, fibrocystic disease of the breast, nasal polyp, Crohn's disease and schwannoma.

Immunohistochemistry

Sections 2 μm thick were cut from formalin-fixed, paraffin-embedded tissue, dewaxed, and immersed in hydrogen peroxide 0.3% in methanol for 30 min to inhibit endogenous peroxidase activity. Sections were predigested at 37°C with pepsin (Sigma, St Louis, MO, USA) 0.1% in HCl 10 mM for one hour (9H3 and anti-tryptase), or protease XXIV (Sigma) 0.05% in Tris-buffered saline (TBS) pH 7.5 for 10 minutes.

Prior to 9H3 and anti-tryptase incubation, some sections were further digested with purified collagenase VII (Sigma) 1 mg/ml in 0.9% sodium chloride for 1 hour at 50°C. All antibodies were diluted in TBS/bovine serum albumin (BSA) 1%, and incubated for one hour at room temperature, or overnight at 4°C (9H3/vWf). Controls included omission of the primary antibody and substitution of an irrelevant monoclonal antibody of the same isotype, or normal rabbit or goat serum where appropriate. For monoclonal reagents peroxidase-conjugated rabbit anti-mouse IgG (Dako) diluted in 50% heat-inactivated human serum was then incubated for 30 minutes, followed by peroxidase-conjugated swine anti-rabbit IgG (Dako). The reactivity with polyclonal antibodies was revealed using swine anti-rabbit or rabbit anti-goat immunoglobulins as bridging reagents followed by rabbit or goat PAP

(Dako) respectively. Extensive washes with TBS followed all antibody incubations. After exposure to DAB substrate, sections were counter-stained with haematoxylin, dehydrated, and mounted in DPX.

For each antibody, positive cells in three medium-power fields (×50 magnification) were counted using a 25-point Zeiss integrating grid. The number of tryptase-positive cells served as a reference for the total number of mast cells.

Double labelling. To best demonstrate the close relationship of type VIII collagen-positive mast cells to vessels, double labelling studies were undertaken on a variety of tissues. After pepsin treatment of sections polyclonal anti-vWf and 9H3 (anti-type VIII collagen) antibody were simultaneously incubated overnight. Application of swine anti-rabbit immunoglobulin (Dako) and biotinylated goat anti-mouse immunoglobulin antibody (Biogenex) was followed by incubation with rabbit PAP and alkaline phosphatase-conjugated streptavidin (Biogenex). The enzymatic reaction products were demonstrated with AEC (peroxidase) and NBT/BCIP (alkaline phosphatase).

Histochemistry

Mast cells were revealed using the fluorescent dye berberine sulphate (Enerbäck 1974), the metachromatic stain toluidine blue (0.5% in 0.5 M HCl), and orthochromatically by a combined Alcian blue/safranin method (Alcian blue 0.36%, safranin 0.18% in Walpole's acetate-HCl buffer, pH 1.42).

Double labelling. After immunohistochemical detection of type VIII collagen with 9H3, as described above, some sections were double-stained with Alcian blue and safranin.

In situ *hybridization*

Oligonucleotide probes based on the published human α-1 collagen VIII sequence (GenBank COL8A1) (Muragaki *et al.* 1991) were purchased from Operon Technologies (Alameda, California, USA), including two anti-sense primers (5′-CCAATTTCCCCTTTCTGTCC-3′, complementary to nucleotides 544–563, 5′-ATGGTATTTCTTTGCCTTTCTTG-3′, complementary to nucleotides 315–337), and one sense primer (5′-GCTTACCATTTCCCTGAGTTCC-3′, nucleotides 42–63). All primers were 3′-end labelled with digoxigenin using the Boehringer-Mannheim oligonucleotide tailing kit (Boehringer-Mannheim, Mannheim, Germany). The manufacturer's instructions were followed, except that the labelling reaction time was reduced to 10 minutes to minimize probe digestion (Crabb *et al.* 1992), and the final ethanol wash was omitted. Formalin-fixed paraffin sections were prepared for hybridization as previously reported (Pringle *et al.* 1989), except that digestion with proteinase K 1.5 μg/ml (Boehringer-Mannheim) was preceded by dehydration through graded ethanol, and post-fixation was followed by a 10-minute incubation in acetic anhydride 0.25% in triethanolamine. Control slides were incubated with RNase A 100 μg/ml (Boehringer-Mannheim) for one hour at 37°C. The hybridization buffer contained sodium chloride 600 mM, Tris-HCl 50 mM pH 7.5, sodium pyrophosphate 1%, EDTA 5 mM, formamide 10%, PEG 6000 5%, polyvinylpyrolidone 0.2%, Ficoll 0.2%, poly-(A) 100 μg/ml and sheared salmon sperm DNA 160 ng/μl. Prehybridization for one hour at 37°C was followed by overnight hybridization at 37°C with 200 ng of either the anti-sense probes, in an equimolar mixture, or the sense probe. Slides were washed at 22°C three times with 2 × SSC for 10 minutes, then once with 0.2 × SSC for 30 minutes. Hybridized probe was detected using alkaline phosphatase-labelled anti-digoxigenin antibody, and NBT/X-phosphate substrate according to the manufacturer's instructions (Boehringer-Mannheim). Slides were counterstained with neutral red, air dried, and mounted in DPX.

Results

Mast cells were revealed in a wide range of tissues using the above profile of antibodies and histochemical techniques. Toluidine blue stained the cells metachromatically, and safranin and berberine sulphate revealed the heparin-containing cells. Many Alcian blue-positive and safranin-positive mast cells stained for type VIII collagen (9H3), as seen in the double-stained section in Figure 1. Sequential sections of urticaria pigmentosa showed mast cells staining with tryptase, chymase, c-*kit* and 9H3, indicating that a proportion of mast cells label for type VIII collagen (Figure 2, A–D). The mast cells examined at all other sites, with the exception of the asthmatic lung and Crohn's disease mucosa, also showed positive staining for type VIII collagen in a subset of mast cells, using 9H3 (Table 1). 6A2 and polyclonal anti-type VIII collagen produced a similar staining pattern and tissue distribution of positive cells, but staining was of lower intensity than that with 9H3. Collagenase pretreatment did not alter mast cell staining for tryptase, but completely abrogated staining

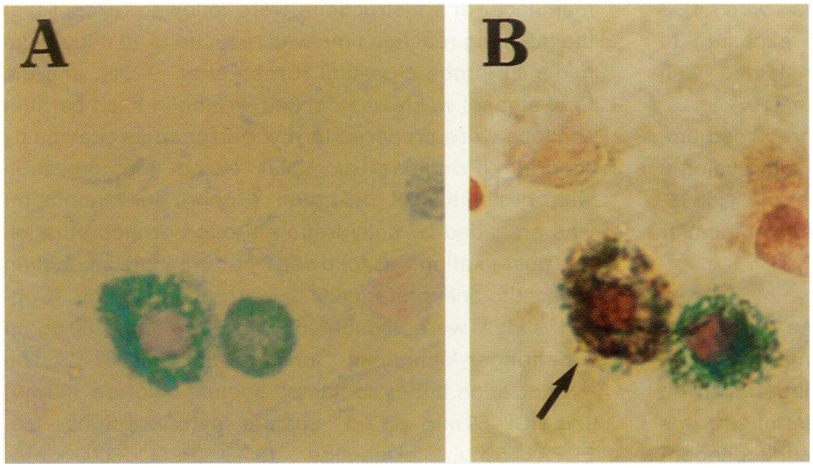


Figure 1. Mast cells in nasal polyp double-stain with Alcian blue and 9H3 (monoclonal anti-type VIII collagen). A, Mast cells staining with Alcian blue in a section of nasal polyp. ×725; B, double-labelling of a mast cell in a section of nasal polyp. The mast cell indicated with the arrow shows Alcian blue-positive cytoplasmic granules and simultaneously stains with 9H3 antibody specific for type VIII collagen. The adjacent mast cell does not express type VIII collagen. ×725.

Figure 2. (Facing page) Urticaria pigmentosa mast cells produce type VIII collagen. A. Anti-tryptase immunohistochemistry. Arrows indicate labelled mast cells. ×210. Inset: the location of a mast cell infiltrate in relation to the epidermis at low power. × 50. *Epidermis. B, Anti-chymase immunohistochemistry on a section consecutive to that depicted in A. Arrows indicate the labelled mast cells. ×210. C, Anti-c-*kit* immunohistochemistry. Specific labelling of mast cells indicated by arrows. ×210. D, Anti-type VIII collagen α-1 chain immunohistochemistry (9H3 monoclonal antibody) on a section consecutive to that depicted in B. ×210. E, *In situ* hybridization with digoxigenin-labelled oligonucleotide probes, for type VIII collagen α-1 chain mRNA. ×210. Blue granules indicate specific message detected within mast cells (arrows). Inset: negative control section treated identically except for pre-treatment with RNase. × 210. F, High-power view of *in situ* hybridization depicted in E. ×500.

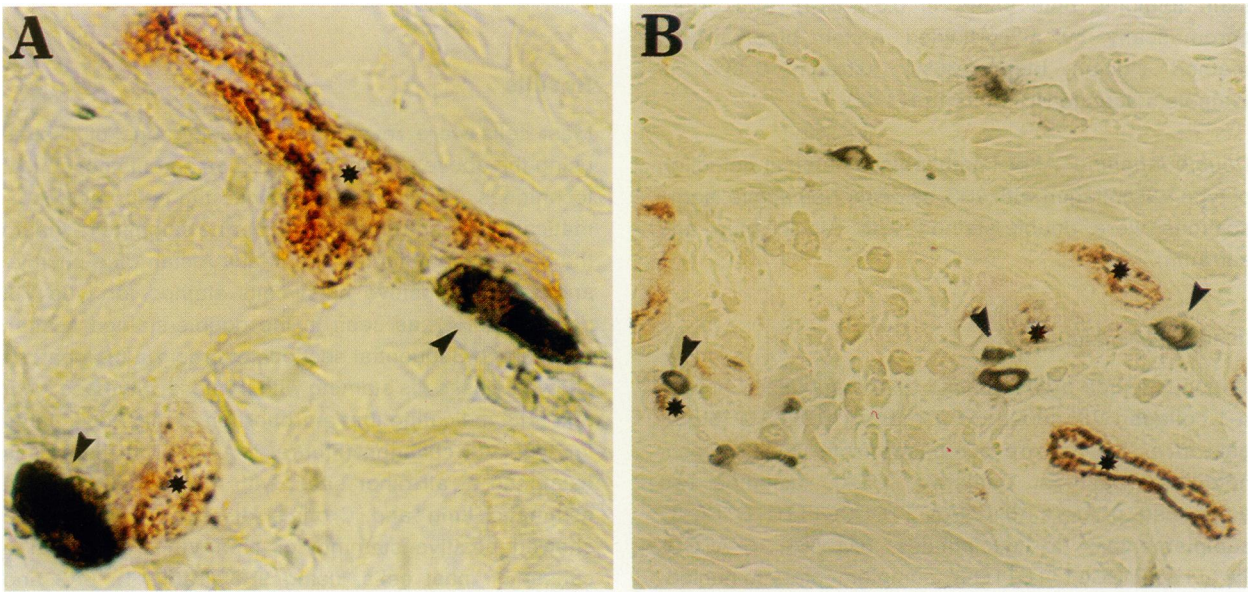
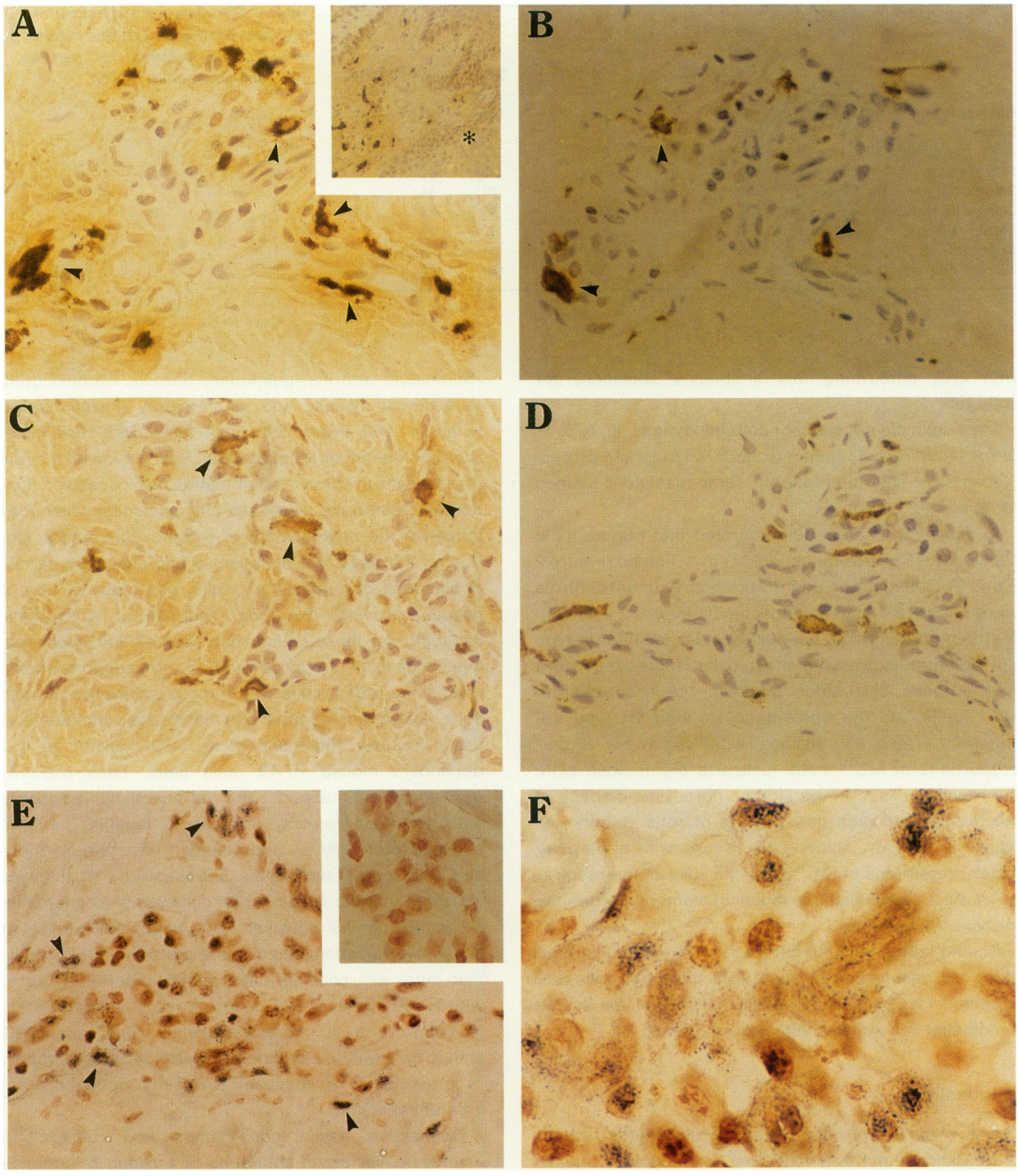


Figure 3. Double labelling for type VIII collagen positive mast cells and vascular endothelium. A, 9H3-positive mast cells (blue-grey; alkaline phosphatase) and von Willebrand factor-labelled vascular endothelium (red-brown; peroxidase) on breast fibrosis tissue. Arrow heads indicate type VIII collagen positive mast cells. *Vessel lumens. ×650. B, Type VIII collagen-positive mast cells (blue-grey staining, arrow heads) and von Willebrand factor stained vascular endothelium (red-brown; lumens indicated by *) on urticaria pigmentosa skin. × 270.


A
B
C
D
E
F

	Chymase			
	+ve (MC^{TC})	–ve (MC^{T})	c-*kit*	Collagen VIII
Fibrocystic disease of the breast	97	3	100	90
Kidney	95	5	85	89
Urticaria pigmentosa	75	25	73	73
Schwannoma	48	52	76	52
Crohn's disease sub-mucosa	72	28	72	54
Crohn's disease mucosa	32	68	43	< 5
Nasal polyp	49	51	52	11
Asthmatic lung	36	64	66	< 5
Idiopathic pulmonary fibrosis	0	100	100	30

Table 1. Percentages of mast cells (tryptase-positive) in various tissues ($n = 2$ for each) staining for chymase, c-*kit*, and collagen VIII (9H3)

with 9H3, confirming this antibody's specificity for collagen.

Mast cells did not stain for collagen types I, III, IV or V, or for laminin-A, s-laminin or heparan sulphate proteoglycan. However, interestingly, some mast cells stained for laminin-B2 chain.

In situ hybridization demonstrated that human mast cells express mRNA for collagen VIII α-1 chain (Figure 2 E and F). Sense probe and RNase pretreatment controls were negative, indicating specificity (Figure 2 E, inset). A striking finding was the perivascular location of type VIII collagen-expressing mast cells. Moreover, there was a loose correlation between the proportion of MC^{TC} cells and the percentage staining for type VIII collagen (Table 1). Tissues with somewhat higher proportions of MC^{TC} cells (urticaria pigmentosa skin, the reactive tissue in the kidney, fibrocystic disease of the breast) had high percentages of cells staining for type VIII collagen. Conversely tissues with apparently lower proportions of MC^{TC} cells (tissues of the respiratory tract, Crohn's disease mucosa) stained infrequently for type VIII collagen.

Double-labelling immunohistochemistry demonstrated the close association of some 9H3 (type VIII collagen) positive mast cells and vessels as stained for by the endothelial cell marker, vWf (Figure 3 A and B).

Discussion

The involvement of mast cells in acute allergic events is well known, but they may also be integral to inflammation, haemostasis, haematopoiesis, angiogenesis, tissue remodelling and tumour development (Claman 1990; Frieri 1992; Galli 1990; 1993; Hawkins *et al.* 1985; Meininger & Zetter 1992). In particular, mast cells are known to be associated with fibrosing conditions and may play a central role in several clinically important chronic inflammatory conditions in man, including scleroderma, rheumatoid arthritis, pulmonary fibrosis, chronic graft-versus-host disease and inflammatory bowel disease (Claman 1990; Frieri 1992; Galli 1990; 1993; Hawkins *et al.* 1985; Meininger & Zetter 1992). Speculation on the role of mast cells in chronic inflammation has focused on their ability to elaborate proteases and cytokines, which may influence the development of extracellular matrix. Our finding that type VIII collagen is expressed by certain populations of human mast cells, especially in fibrotic tissues, provides the first evidence of a synthetic capability for these cells in the construction of connective tissue *in vivo*. Previous work *in vitro* supports such a role, since rodent cells with the characteristics of mast cells can synthesize basement membrane components in tissue culture. Thompson *et al.* (1991) examined IL-3 dependent mouse PT-18 mast cells, bone marrow-derived mouse mast cells and rat basophilic leukaemia cells for their capacity to express laminin, heparan sulphate proteoglycan and collagen IV, using Northern and Western blot techniques. The Western blot analyses indicated that the cells secrete collagen IV and laminin-B1 and -B2 chains. However, no immunohistochemical or *in situ* hybridization studies were performed. It is known that there are considerable species differences as to which cell types elaborate the various laminin chain isoforms. It is of interest, therefore, that laminin-B2 should be identified by different methods to be a product of mast cells or mast-cell-like cells obtained from different species.

Connective tissue construction and remodelling involves a variety of collagens in a range of complex processes, and type VIII collagen is believed to contribute by forming a molecular scaffolding in the extracellular matrix (Rosenblum *et al.* 1993). The fact that human mast cells *in vivo* did not demonstrate expression of collagen types other than type VIII, might suggest that the production of a scaffold is their primary direct contribution to extracellular matrix construction. *In situ*

mast cell proliferation and type VIII collagen production may be early events in healing and fibrosis that are later assisted or completed by fibroblasts and their interstitial fibrillar collagen products.

Mast cells are frequently found around blood vessels (Galli 1993), and we observed that many of these perivascular mast cells expressed type VIII, but not interstitial (types I, III, and V) collagens (Figure 3 A and B). Synthesis of type VIII collagen is strongly associated with angiogenetic processes *in vitro* (Iruela-Arispe *et al.* 1991; Rooney *et al.* 1993). The precise role of collagens in angiogenesis remains unclear, but they may be a necessary substratum for the ingress of endothelial cells (Iruela-Arispe *et al.* 1991). Although our data do not directly bear on the issue in a functional sense, it seems likely that type VIII collagen produced by perivascular mast cells is involved in angiogenesis.

Mast cells have been shown to have extraordinary phenotypic plasticity and heterogeneity under the influence of different microenvironments (Galli 1990; 1993). At different anatomical sites, and even a single site, mast cells demonstrate substantial differences in mediator content, in sensitivity to agents that induce activation and mediator release, and in their responses to pharmacological agents. Similarly, many histochemical methods for localizing mast cells stain only a proportion of the total mast cells present, especially in formalin-fixed tissue. Furthermore, several of these methods are not specific for mast cells (Walls *et al.* 1990). Tryptase, however, is expressed by all mast cells in human tissue and not by other cells (Galli 1990; Irani *et al.* 1986; Walls *et al.* 1990). We identified mast cells in human tissue using a monoclonal antibody to tryptase and employed other histochemical and immunohistochemical methods to confirm mast cell identity and to obtain additional information on mast cell phenotype. As expected, expression of these other markers varied from cell to cell and from site to site. Proportions of mast cells expressing chymase (MC^{TC} phenotype) in each tissue were in agreement with those previously reported (Table 1) (Irani *et al.* 1986). c-*kit* was not identified on all mast cells: in some tissues more than half the mast cells present did not express this receptor at detectable levels (Table 1). Previous work (Mayrhofer *et al.* 1987) also found c-*kit*-negative mast cells in human tissue, although the histochemical method used in their studies to mark mast cells was not mast cell specific. Differential expression of c-*kit* by mast cells in individual tissues may result from their different stages of differentiation or activation (Galli *et al.* 1993; Mayrhofer *et al.* 1987). This is the first published report of the detection of c-*kit* in paraffin-embedded human tissue using a monoclonal antibody, and it is also possible that formalin fixation may reduce the level of staining.

Type VIII collagen expression by mast cells also varied from cell to cell and from tissue to tissue. Staining for type VIII collagen was not strictly associated with either Alcian blue or safranin positivity, indicating expression of type VIII collagen by mast cells is not necessarily linked to the presence of biogenic amines or heparin. The highest proportions of type VIII collagen-positive mast cells were seen in fibrotic specimens (Table 1). Those tissues with the highest numbers of MC^{TC} cells also had the most cells expressing type VIII collagen (Table 1). The capacity of mast cells to express chymase may well be associated with the ability to synthesize and secrete type VIII collagen in connective tissues but no direct correlation can be drawn from the available data.

Clearly these data further support the contention that a wider role for mast cells in human pathology should be considered (Galli 1990; 1993). Although resident mast cells are known to proliferate at sites of inflammation, injury and fibrosis (Galli 1993), and to produce multiple pro-inflammatory cytokines (Galli *et al.* 1991), proteases (Nadel 1991), and other mediators (Stevens & Austen 1989), their precise functions in these circumstances are not understood. This communication, establishing a connection between mast cell proliferation in inflammatory and fibrotic conditions in man, and the release of type VIII collagen, a molecule known to have angiogenetic and fibrotic associations, provides important new information on the issue. Future studies will need to establish a functional link between the capacity of mast cells to produce type VIII collagen, and the contribution of that product to normal and pathological human biology.

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